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A microscopy-based approach for determining growth probability and lag time of individual bacterial cells

Lena Fritsch [a], Abirami Baleswaran [a], Hélène Bergis [a], Adrienne Lintz [c], Erwann Hamon [c], Valérie Stahl [c], Jean-Christophe Augustin [a,b], Laurent Guillier [a,*]

[a] French Agency for Food, Environmental and Occupational Health & Safety (Anses), Laboratory for Food Safety, Université Paris-Est, Maisons-Alfort F-94701, France
[b] Ecole Nationale Vétérinaire d'Alfort, 94700 Maisons-Alfort, France
[c] Aérial, Technical Institute of Food Industry, Parc d'innovation, 250 rue Laurent Fries, 67400 Illkirch, France

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ABSTRACT

The development of relevant predictive models for single-cell lag time and growth probability near growth limits is of critical importance for predicting pathogen behavior in foods. The classical methods for data acquisition in this field are based on turbidity measurements of culture media in microplate wells inoculated with approximately one bacterial cell per well. Yet, these methods are labour intensive and would benefit from higher throughput. In this study, we developed a quantitative experimental method using automated microscopy to determine the single-cell growth probability and lag time. The developed method consists of the use of direct cell observation with phase-contrast microscopy equipped with a 100× objective and a high-resolution device camera. The method is not a time-lapse method but is based on the observation of high numbers of colonies for a given time. Automation of image acquisition and image analysis was used to reach a high throughput. The single-cell growth probabilities and lag times of four strains of *Listeria monocytogenes* were determined at 4 °C. The microscopic method was shown to be a promising method for the determination of individual lag times and growth probability at the single-cell level.

1. Introduction

In the field of predictive microbiology, mathematical models are developed since decades to describe the bacterial behavior in foods. Multiple (experimental and biological) factors may affect the bacterial behavior hampering the precise assessment of bacterial responses, especially in harsh environmental conditions (Aryani, Den Besten, Hazeleger, & Zwietering, 2015; den Besten, Aryani, Metselaar, & Zwietering, 2016). Besides the variability among different strains of a species, the heterogeneity within a population should be taken into account during growth or inactivation predictions (Aspridou & Koutsoumanis, 2015; Koutsoumanis & Lianou, 2013). Two main approaches are usually adopted in an experimental design to assess bacterial phenotypes: the "*population*" and the "*individual cells*" ones. In the first case, the behavior is modeled without taking into account the variability of the cells that constitute the population. Yet, not all bacteria in a clonal population react in the same way to environmental changes (Koutsoumanis & Aspridou, 2017; Koutsoumanis & Lianou, 2013). Indeed, some individual cells can show extreme tolerance to a given stressor or extreme sensitivity compared to the majority of the cells within the population. A particular attention should be paid to the bacterial cells showing higher resistance to stressful environmental conditions (Aguirre & Koutsoumanis, 2016; Aspridou & Koutsoumanis, 2015; Margot, Zwietering, Joosten, & Stephan, 2016). This enhanced resistance can promote the persistence of bacterial pathogens (e.g. *L. monocytogenes*) in food processing plants and the colonization of new environments, increasing the risk of food contamination (Pascual, Robinson, Ocio, Aboaba, & Mackey, 2001). In addition, a shorter lag time has been reported for bacterial cells with enhanced resistance to environmental stresses in comparison to sensitive ones (Margot et al., 2016).

Food matrices are often contaminated with low levels of bacteria (Ross & McMeekin, 2003). Thus, application of the single-cell approach offers the advantage of generating more realistic contamination scenarios. The "*individual cells*" approach considers the individual behavior of each cell within a bacterial population in an independent manner.

* Corresponding author.
E-mail address: laurent.guillier@anses.fr (L. Guillier).

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Data acquisition at the single-cell level requires the establishment of methods capable of isolating each bacterial cell and at the same time to generate large data sets (Swinnen, Bernaerts, Dens, Geeraerd, & Van Impe, 2004). For the determination of single-cell lag time, different methods are available. The most commonly practiced rely on the measurement of the optical density in broth media over time (Smelt, Otten, & Bos, 2002). This technique requires that one cell develops a high number of generations to reach the detection level for turbidity measurement ($\sim 10^7$ cells/ml). The determination of lag time based on this method requires the fine determination of the concentration of cells at the detection limit (Métris, George, Peck, & Baranyi, 2003). Nevertheless, there are certain weaknesses in this indirect approach, especially under particular experimental conditions (e.g. at temperatures close to bacterial growth limits the experiments will take a long time). In addition, the fact that this approach is conducted in broth media makes it harder to transfer results into solid food matrices. The microenvironment of planktonic cells is indeed quite different from that of immobilized cells in colonies, thus the bacterial growth capacity might differ greatly (Verheyen et al., 2019). In case of pathogens' growth prediction, the improvement of knowledge on immobilized growth is of greater concern to avoid over- or under-estimations (Skandamis & Jeanson, 2015). Other indirect methods to estimate the individual lag time of bacterial growth on solid media have been published (Guillier, Pardon, & Augustin, 2006; Levin-Reisman, Fridman, & Balaban, 2014). These authors proposed a method based on image analysis of the bacterial colony growth on agar, where the lag times are estimated by detection times required to form macroscopically visible colonies. Mertens et al. (2012) published a method based on a similar approach, but the growth was monitored through the measurement of OD of colonies (Mertens, Van Derlinden, & Van Impe, 2012). In this case, the throughput was improved with the use of 48 well-plates. However, all these approaches are presenting the same weakness as the indirect method in broth regarding the time required to reach an observation level, especially under conditions close to growth limits.

In order for the aforementioned limitation to be overcome, approaches based on the use of a gel cassette in combination with image analysis to study the growth parameters of bacterial single colonies constitute an interesting starting point (Brocklehurst, Mitchell, & Smith, 1997; Skandamis, Brocklehurst, Panagou, & Nychas, 2007). In addition, some direct methods have been proposed based on microscopy, such as the flow chamber technique and the time-lapse microscopy (Elfwing, LeMarc, Baranyi, & Ballagi, 2004; Koutsoumanis & Lianou, 2013). Elfwing and colleagues' flow chamber technique is based on the monitoring of the consecutive divisions of a single cell attached to a solid surface (Elfwing et al., 2004; Niven, Fuks, Morton, Rua, & Mackey, 2006). On the other hand, Koutsoumanis and Lianou (2013) presented a less complex system to study the single-cell lag time by contrast phase microscopy. This method allows a direct follow up from one cell to a microcolony in real-time. The growth of several cells present in the same microscopic field can be recorded as long as the cells are initially well apart from each other. Only one microscopic field can be recorded per experiment.

Besides the single-cell lag time, it is essential to explore the impact of the growth probability on the outcome of predictive models and thus, on the exposure assessment part of microbiological risk assessment. Augustin and Czarnecka-Kwasiborski (2012) studied the single-cell growth probability of *L. monocytogenes* under different conditions (i.e. temperature, pH and water activity) in broth media by using most probable number (MPN) approach. However, drawbacks of this method are the long experiment duration (i.e. 3 months) and the great uncertainty of concentrations estimated with the MPN method. In this perspective, the objective of this study was to develop a quantitative experimental investigation to determine the single-cell growth probability and lag time of different *L. monocytogenes* strains, using a time-efficient and automated microscopy method.

Table 1
Metadata of *L. monocytogenes* stains.

Isolate	Origin	T_{min}
SOR100	Sausage	−2.52 °C
AER101	Dairy	−0.15 °C
Lm14	Meat processing plant environment	−0.86 °C
O228	Shrimp	unknown

2. Material and methods

2.1. Strains

Four strains of *L. monocytogenes* were tested for establishing the herein developed method (Table 1). They were selected based on their phenotypic and genetic diversity. Three of them (i.e. SOR100, AER101 and Lm14) were part of Symp'Previus project (Couvert et al., 2010) with known cardinal temperatures (i.e. T_{min}). The O228 strains was isolated in shrimp (Palma et al., 2020). It has not been characterized for cardinal temperatures. Before use, the strains were stored in cryobeads at −80 °C.

2.2. Preparation of inocula

The preculture of each strain was carried out by inoculating a cryobead in 10 ml tryptone soy broth (TSB, Oxoid, UK). After 7 h at 37 °C, 0.1 ml was transfered to 9.9 ml fresh TSB. The dilution was then incubated for 17 h at 37 °C. Following, successive dilutions were made in tryptone salt diluent (TS, Biomerieux, UK) to obtain a final concentration of 10^5 CFU/ml.

2.3. Preparation and storage of the microscope slides

Glass slides were prepared by placing 200 µl of melted tryptone soy agar (TSA, Oxoid) in the middle of the slide. After cooling, 10 µl of the diluted inoculum were pipetted on the solid layer TSA. The samples were dried in a laminar flow hood for 5 min and then covered with a glass coverslip. Following, the prepared glass slides were put in a sealable box and were stored in the incubator at 4 °C. At each point of measurement, the required number of slides were pulled out for analyses. Before starting the microscopic observation, coverslips were fixed with silicone.

2.4. Microscopic observation of cells

For the microscopic observation of the cells, a motorized phase-contrast microscope (Nikon, Eclipse Ni-E) was used in combination with a 100 × objective and a high-resolution camera (Nikon, DS-Fi3). Software from Nikon (NIS-Elements, version 4.60) was applied for automatically capturing images in two different areas, each with a surface of 0.25 mm^2, corresponding to 88 images per area. For each defined area, the capture of images was systematic along a virtual grid defining 88 distinct fields. Images were taken even if there are no cell, object inside the optical field.

2.5. Image analysis procedure

The images were captured in TIFF format. Images were then analyzed with Matlab (R2018b) including the Image Processing Toolbox™. The applied image analysis procedure was previously adapted from Guillier and colleagues (Guillier et al., 2006). For object recognition, a threshold was estimated to separate pixels associated to bacterial cells from the TSA background pixels. Because of the variation in thickness of the TSA, the threshold was adapted for each of the image series. Based on the threshold, intensities associated to background were displayed in black and those corresponding to cells in white by using the ind2gray and im2double functions of Matlab (Fig. 1B). Following, two

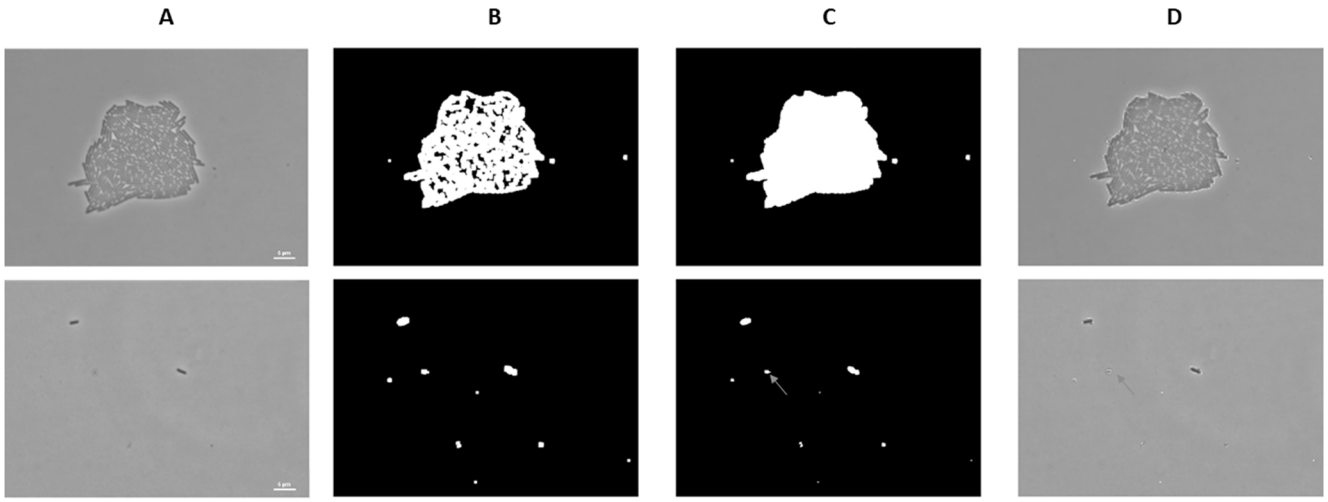

Fig. 1. Different process steps of image analysis used to identifies cells and micro colonies. A) Captured images; B) Black and white contrast; C) Gap closing; D) Detection of objects above the given pixel size threshold defining a cell minimal size. A number is automatically assigned to detected objects. The arrow is highlighting an example of a filtered object according to its size.

morphological operations were applied using bwmorph Matlab function. One to bridge the previously unconnected pixels and another for gap closing (Fig. 1C). Images with no objects or objects below the size of one cell were automatically removed from the analysis. Images with more than three cells or colonies were discarded to avoid bias due to interactions. The detected objects were filtered out when smaller than a single *L. monocytogenes* cell (e.g. arrow in Fig. 1D bottom).

As last step of the procedure, each detected object and the relative number of pixels were exported into a csv file.

2.6. Growth curve at population level

In order to estimate the single-cell lag time and growth probability, the population growth parameters are needed. For the determination of growth parameters of each tested strain at 4 °C, a total of 20 tubes containing each 50 ml TSA were inoculated with 200 µl of the 10^5 CFU/ml suspension to obtain a final concentration of 10^3 CFU/ml. Kinetics were characterized with a minimum of 20 data points by plate counting. The primary model of Baranyi & Roberts (1994) was fitted to the collected growth data and growth kinetic parameters (i.e. lag time and maximum specific growth rate) were estimated using the nlsMicrobio package (Baty & Delignette-Muller, 2004) Three repetitions were carried out.

2.7. Determination of growth probability

The individual cell growth probability was determined by comparing the number of single-cells (*ns*) and number of micro colonies (*nc*), after incubation at 4 °C. The probability of growth was simply estimated with the following relation: ns/(ns + nc). This calculation is possible as long as the micro-colonies have not been merged during their growth. The chosen density of inoculation corresponded to a target of 25 cells per area, that is an average of one cell every three fields. It permitted to limit the probability of merging of micro colonies.

The length of incubation time was chosen to ensure that the individual cells that did not initiate growth at that stage have a low probability to still be in lag phase. The relationships proposed by Guillier and Augustin (2006) were first used to estimate the distribution of physiological state, measured through the work to be done values (h_{0i}), according to the lag time duration observed at population level. The 95th percentile of the estimated h_0 values ($h_{0i\text{-}95}$) was used to estimate the incubation time (T_{prob}) with the following relation $h_{0i\text{-}95}/\mu_{max}$. Thus at T_{prob}, the probability that an observed single-cell is still in the lag phase is lower than 5%.

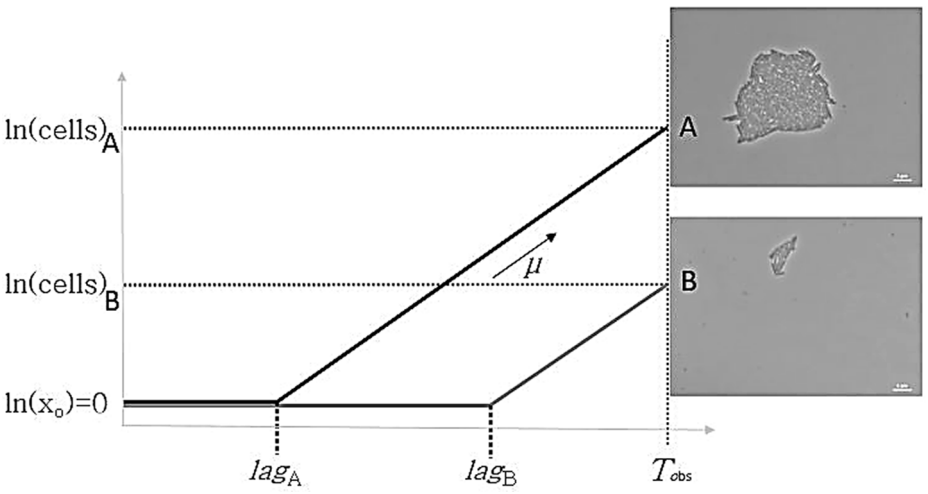

Fig. 2. Schema of the principle to determine single-cell lag times according to the size of microcolonies. Images of micro colonies are captured at T_{obs}. Given the maximum specific growth rate, the lag time duration of the cells that generated the microcolonies can be interpolated.

Table 2
Population growth parameters of the four tested strains with their 95th confidence interval [0.025 0.0975].

	SOR100	AER101	Lm14	O228
Lag time (lag in h)]	58 [18 94]	44 [23 55]	42 [886]	28 [10 43]
maximal specific growth rate (μ_{max} in h^{-1})	0.044 [0.039 0.052]	0.049 [0.047 0.052]	0.038 [0.035 0.045]	0.043 [0.039 0.045]
Work to be done (μ_{max} ·lag) h_0	2.5	2.2	1.6	1.2

The uncertainty of the observed probability of growth at T_{prob} was calculated by using the beta distribution (Vose, 1998):

Beta((1-ns) + 1, nc + 1)

The uncertainty intervals were used to test if there were significant differences between strains.

2.8. Individual lag time determination

The estimation of single-cell lag time is based on the vertical distribution method (d'Arrigo et al., 2006). This method relies on the distribution of numbers of cells observed in different samples (each sample being inoculated with one cell) after a given incubation time. The distribution of lag times of the cells is then transposed with the help of maximum growth rate.

The individual cell lag time lag_i was thus determined using the time of observation T_{obs}, the cell number per micro colony (ln(x)) and the growth rate (μ) of the corresponding strain based on number of cells in the microcolony and the growth rate (Fig. 2).

$$lag_i = T_{obs} - \frac{\ln(X)}{\mu}$$

Twenty samples were stored in the incubator (4 °C) during the experiment. Then the samples were analyzed at three different T_{obs}, to ensure at least that one point of analysis corresponds to a time where most of the cells have already left lag-time, but without starting to grow in the third dimension (double-layer and multi-layer). After microscopic observation, which was carried out at room temperature in less than 20 min, the samples were destroyed. For each measurement point, new samples were used. The number of cells per colony was estimated based on the number of pixels per colony and a correlation function.

The lognormal distribution was fitted to the estimated individual lag time distribution and the fitdistcens function from the fitdistrplus package (Delignette-Muller & Dutang, 2015). Parametric bootstrap was used to assess credibility interval on quantiles characterizing the median of individual lag times. The *rogme* package (https://github.com/GRousselet/rogme) was used to compare all deciles.

3. Results

3.1. Growth rates

Table 2 shows the population growth parameters at 4 °C for all tested strains. The strain SOR100 had the longest lag time (58 h) compared to the others. Contrary, the strain O228 showed the shortest lag time of 28 h.

Regarding the maximal specific growth rate, AER101 (0.049 h^{-1}) was the fastest while Lm14 (0.038 h^{-1}) the slowest strain under the

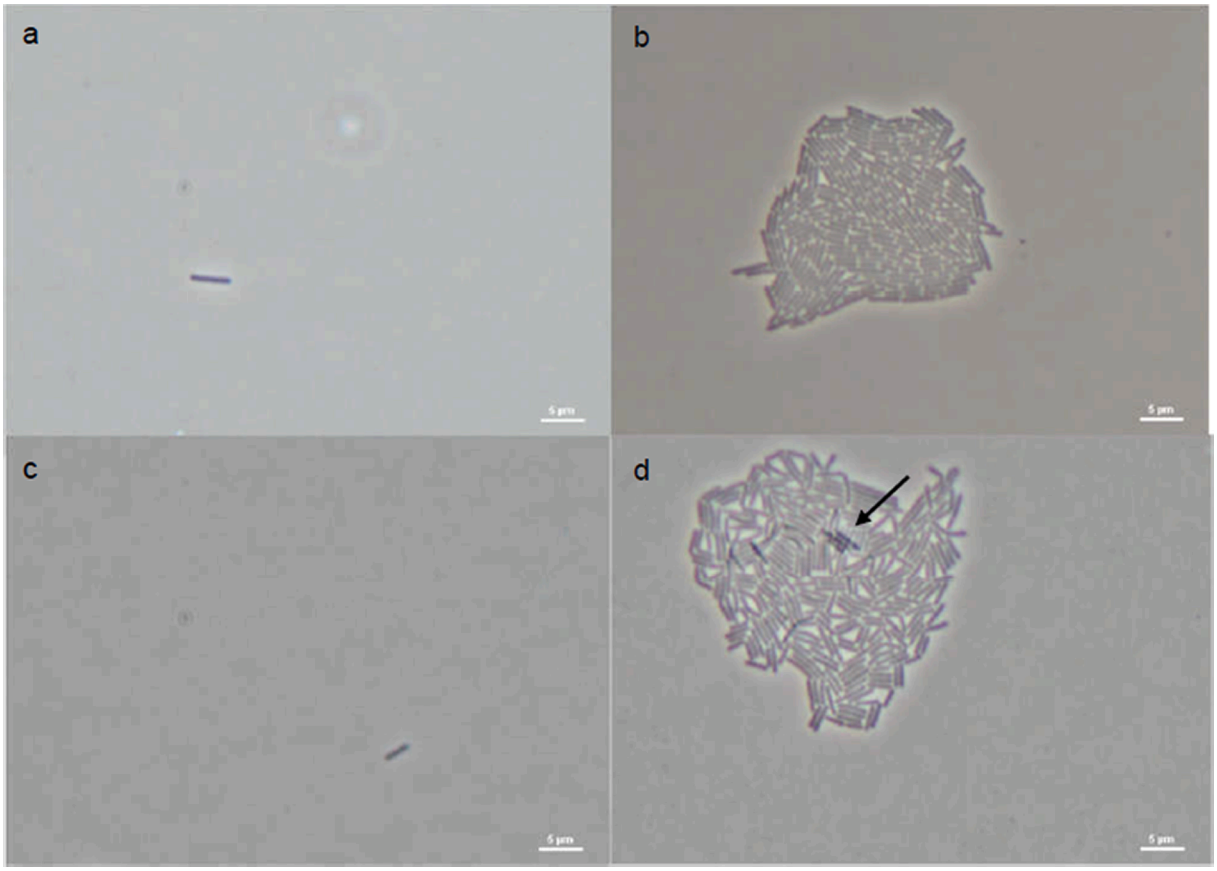

Fig. 3. Examples of *L. monocytogenes* images obtained with phase-contrast microscope (Nikon, Eclipse Ni-E) in combination with a 100 × objective and a high-resolution camera (Nikon, DS-Fi3) after various time of incubation on agar surface at 4 °C. ,. a) Cell of AER 101 strain after 3 days. b) Microcolony of AER101 at T_{obs} (time used for lag time determination). c) and d) Micro colonies of SOR100 strain captured at T_{prob} (time used for growth probability determination). The arrow is indicating a double-layer.

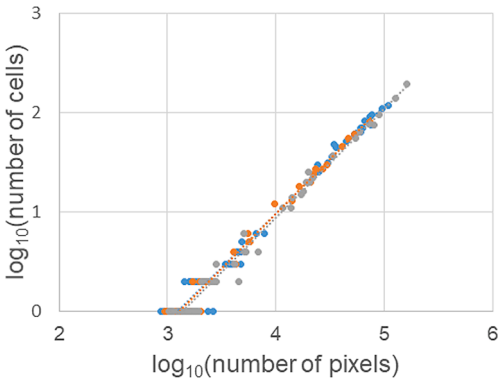

Fig. 4. Relationship between the number of cells per colony and the number of pixels for *L. monocytogenes* strain O228. The linear regression lines are adjusted with several series of points corresponding to the different repetitions of the experiment; blue = R1, red = R2 and green = R3. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

tested conditions. It is worth to notice that SOR100 had the highest h_0 value of 2.5 and O228 the lowest value of 1.2.

3.2. Determination of cell numbers in the captured images

Fig. 3 presents four examples of the captured images. Fig. 3a shows a single-cell of strain AER101 after 3 days at 4 °C. The cell had already initiated cell elongation (compared to cells at day 0). Fig. 3b displays a microcolony of AER101 at T_{obs} for lag time determination. Fig. 3c and d were both captured at T_{prob}. The single-cell (Fig. 3c) was not able to grow under the tested conditions, whereas the Fig. 3d presents an example of a microcolony (with double-layer) developed from a single-cell of the same strain (SOR100).

In order to determine the number of cells in a microcolony from pixel size, a calibration curve was established for each strain. An example is presented in Fig. 4. Especially, the number of pixels that corresponds to single cells are quite variable, ranging from 875 to 2624 pixels. In contrast, for the number of pixels corresponding to micro colonies a lower variability can be observed.

3.3. Growth probability

Fig. 5 shows the different experiments carried out for the four strains. The variability observed among experiments was smaller than the variability observed among strains. The different experiments were thus merged in a single dataset for each strain. The comparison of individual cell growth probability according to the strain was thus carried out on merged dataset (Fig. 6). More than 200 observations were used to determine the growth probability for each strain.

The strain SOR100 has the highest growth probability with 81% [76%, 85%]. The lowest growth probability is represented by strain O228 with 18% [17%, 22%]. For strain Lm14 a growth probability of 57% [51%, 63%] was determined. It is the second lowest of the tested strains and significantly different from AER101, SOR100, and O228. The growth probabilities of the strains SOR100 (81% [76%, 85%]) and AER101 (73% [67%, 78%]) are not significantly different.

3.4. Individual lag times of single-cells

Fig. 7 shows the difference between the different experiments carried out for the four strains. The variability observed between experiments was smaller than the variability observed among strains. The different experiments were thus merged in a single dataset for each strain. The comparison of individual lag times according to the strain was thus carried out on merged dataset.

The range and variability of individual cell lag times is shown on Fig. 8A. The Fig. 8B presents the pairwise comparison of deciles of the individual cell lag times. Regarding Fig. 8B1 B3 and B5, O228 strain shows significantly lower deciles. Individual lag times of strains Lm14 and SOR100 cannot be distinguished (Fig. 8B2). The first fifth deciles of strain AER101 are similar to SOR100 and Lm14. The other deciles for this strain are significantly lower compared to SOR100 and Lm14 (Fig. 8B4 and B6).

4. Discussion

The method presented in this study allows to generate a high amount of observations for single-cells growing under unfavorable growth conditions in order to estimate lag time and growth probability. Compared to indirect methods where the duration of the experiment is dependent on the time needed for a cell to generate enough generations according to the detection threshold, the proposed microscopy method saves time by reducing the number of generations to obtain lag time and growth probability results. For instance, Augustin and Czarnecka-Kwasiborski (2012) studied the single-cell growth probability in broth based on MPN approach. Under cold conditions (5 °C), their experiments took up to three months to determine the growth probability of *L. monocytogenes*. The herein developed method was more than six times faster (less than two weeks) at similar temperature conditions.

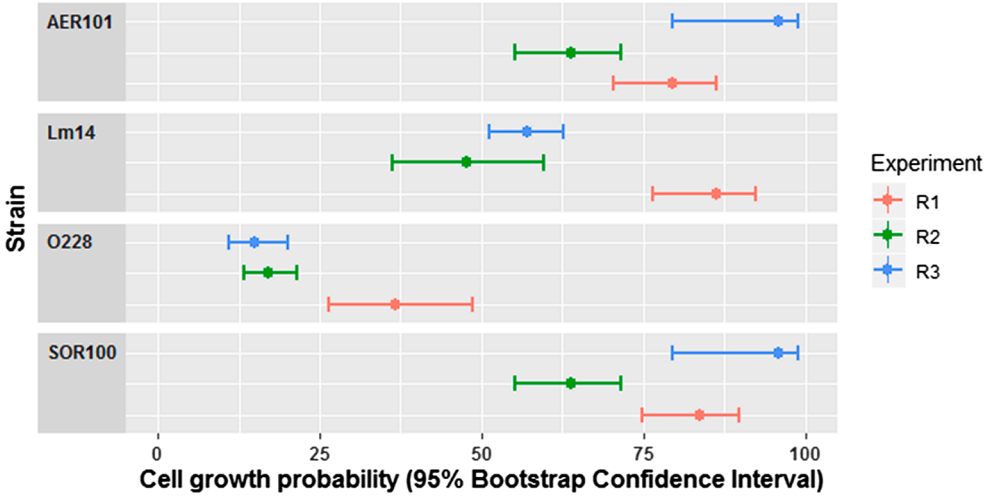

Fig. 5. Cell growth probability at 4 °C of the four *L. monocytogenes* strains tested according to the different repetitions.

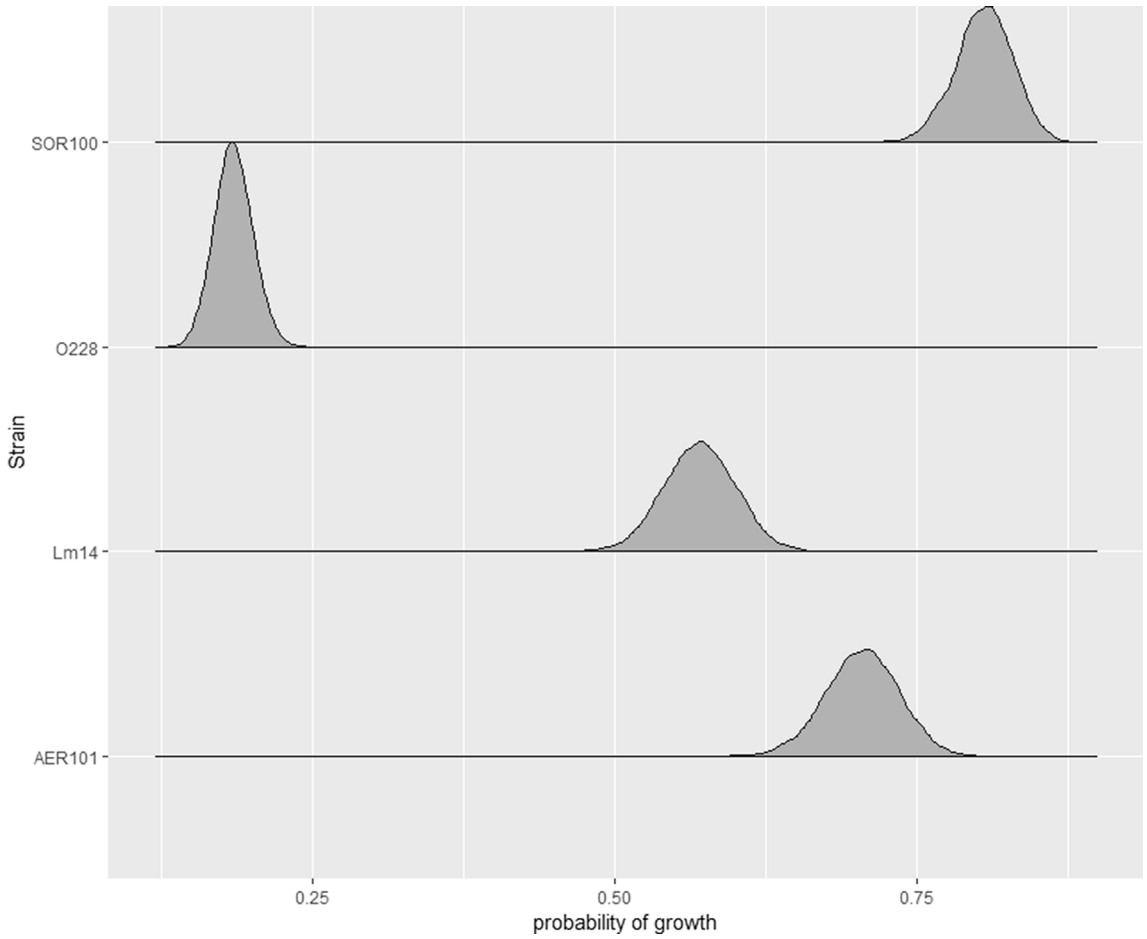

Fig. 6. Uncertainty distribution of individual growth probability of the four strains of *L. monocytogenes* at 4 °C on TSA agar.

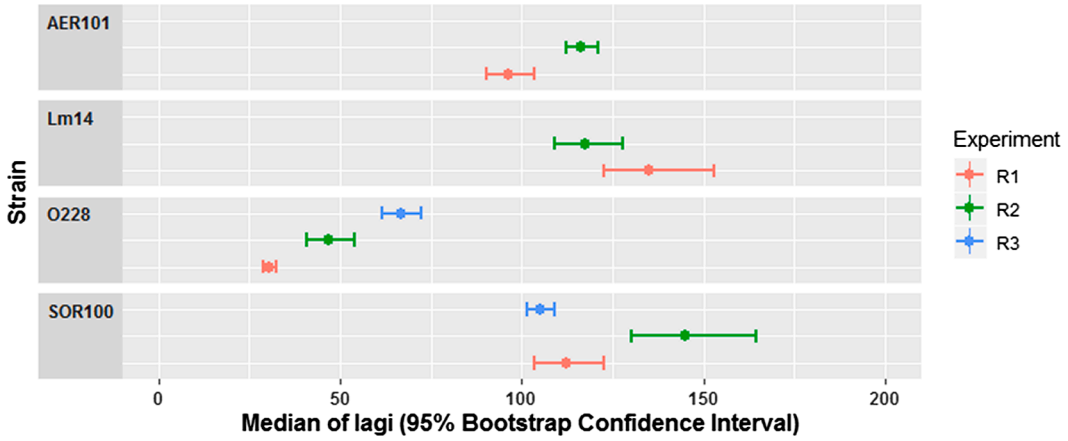

Fig. 7. Median of the individual cell lag times of the four *L. monocytogenes* strains at 4 °C on TSA agar according to the different repetitions of the experiment.

Compared to indirect methods, microscopy-based approaches appear to be much faster also regarding the single-cell lag time determination (Amselem, Guermonprez, Drogue, Michelin, & Baroud, 2016; Levin-Reisman et al., 2010). Francois and colleagues inoculated microtiter plates with approximately one cell per well. They determined about 100 individual lag phases for each set of conditions (Francois et al., 2005). Based on a relation between optical density and cell count, they were able to estimate single-cell lag time. However, 24 generations are needed for the initial cell to reach the lower limit for optical density measurement (around 10^7 CFU/ml) (Francois et al., 2005). Microscopy methods counteract the limitations related to the detection limit since each single-cell can be directly observed. Hence, between these methods a difference in experimental duration is observed especially at low temperature conditions where generation times can be particularly long.

Koutsoumanis and Lianou (2013) proposed a direct method for monitoring single-cell colonial growth dynamics on agar media based on time-lapse microscopy (Koutsoumanis & Lianou, 2013). Compared to optical density measurement and its correlation to obtain the cell number (Francois et al., 2005), the advantage of this method is the direct following up of cells. Even though the time to determine the lag time of

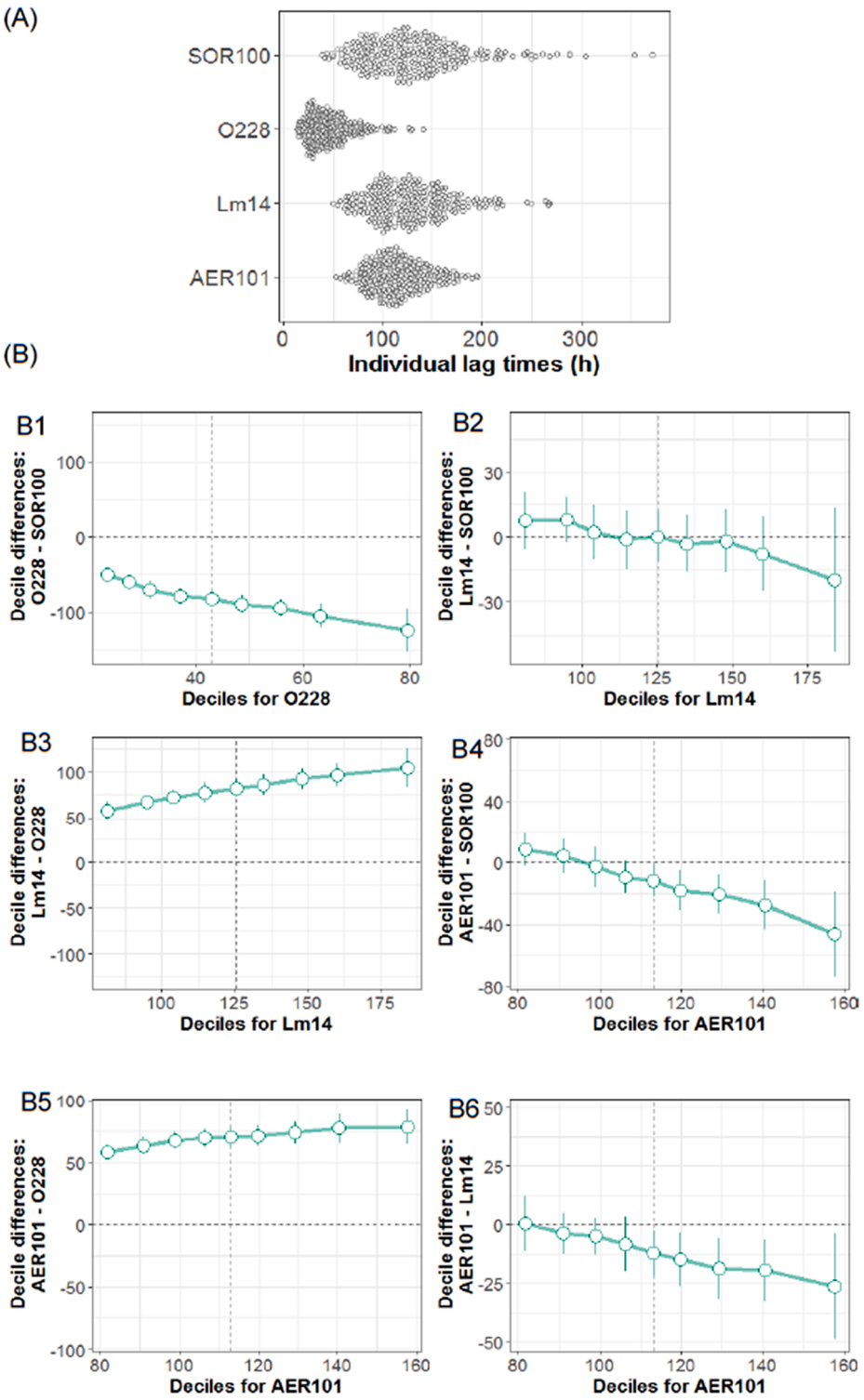

Fig. 8. A) Random individual lag times at 4 °C drawn from fitted lognormal distributions for the four *L. monocytogenes* strains. B) Pairwise strain comparisons of individual lag time deciles.

one cell is shorter, with this approach it is possible to track only few cells at the same time. In contrast, the novel approach herein presented permits to investigate many slides at the same time resulting in a higher amount of data achievable in comparison to the time-lapse approach. However, the drawback of the method is that different slides are recorded at each time of measurement, which presents a source of uncertainty. Both these microscopy approaches permit the following up of cells on agar. Due to the fact that planktonic cell behave in a different manner compared to immobilized cells, the use of agar better simulates the food matrix (Skandamis & Jeanson, 2015). Moreover, the colonial growth dynamics of bacteria on solid foods can be influenced by matrix-specific interactions and gradients within or around the micro colony (Augustin, Ferrier, Hezard, Lintz, & Stahl, 2015; Ferrier, Hezard, Lintz, Stahl, & Augustin, 2013; Malakar, Barker, Zwietering, & Van't Riet, 2003; Walker, Brocklehurst, & Wimpenny, 1997). The method proposed by Elfwing and colleagues permits to study bacterial mother cells

attached to a solid surface and its daughter cell, however, it is not able to observe colonial growth (Elfwing et al., 2004) as the case of agar-based methods. Another huge advantage lies in the possibility to apply dynamic experimental conditions to estimate the growth probability. The slides can be easily stored in incubator with changing temperature to study the effect of temperature flows on the bacterial growth ability, for example to reproduce possible cold chain scenarios.

The presented results for the growth probability are different from those obtained by Augustin and Czarnecka-Kwasiborski (2012). They used in their study the strain Lm14 and obtained at 5 °C a growth probability of 14% (95% Cl 7–20%), whereas in the present study the probability of growth for the same strain was 57% (95% CI 51–63%) at 4 °C. Considering that the uncertainties have been correctly characterized with experimental reproducibility, a potential explanation lies in the different experimental approaches, which is based in one hand on microtiter plates with broth media and in the other hand on growth on surface agar. In this system, the oxygen transfer to the bottom of the wells is limited due to missing agitation, likely impacting the growth probability.

Regarding the results for single cell growth probability, a huge variability can be observed among different *L. monocytogenes* strains. More precisely, the growth probability is highly variable between SOR100 and O228 (18% and 81% respectively), highlighting that growth variability could have an impact on exposure assessment. Interestingly, the strain SOR100 with the lowest minimal growth temperature (-2.52 °C) showed the highest growth probability at 4 °C and seems thus more resistant to low temperatures than the other tested strains. However, more strains should be investigated for such a trend to be ascertained.

In case of the individual lag time (without prior stress exposure), Francois and co-workers observed a mean of 40.1 h in broth at 4 °C (Francois et al., 2005). A similar result was observed for the strain O228 in the present work. However, the median of individual lag times of O228 was about 70 h shorter compared to the three other tested strains. The longer lag times compared to those in the study of Francois and co-workers could be explained by the use of a single strain and/or a different culture media (broth *vs* agar). Indeed, Koutsoumanis and colleagues showed that growth limits of *L. monocytogenes* are affected by the use of solid or liquid media (Koutsoumanis, Kendall, & Sofos, 2004). They observed a lower growth probability when bacteria are grown on a solid surface compared to suspensions. They supposed that modifications of the local environment can lead to a reduced metabolic activity in some regions of the colony.

Although the main purpose of this study was to set up an efficient method for high throughput phenotypic data generation, unexpected differences were observed among the experimental replicates of the fours tested strains. Even when the observed variability between experiments was smaller than the variability among strains, the variability could be decreased by the standardization of datasets to correct the sources of variability between experiments as applied by Guillier et al. (Guillier, Pardon, & Augustin, 2005).

5. Conclusion

This work describes a novel approach to study individual lag times as also single cell growth probability with a high throughput data generation in a short time. This is a promising method for the study of bacterial cells' behaviors at different environmental conditions (e.g. static or dynamic), during colonial growth starting from a single cell. Findings from this study demonstrate that the herein presented approach allows to obtain a high amount of data faster than using indirect growth monitoring approaches or time-lapse microscopy methods, even at unfavorable growth conditions. In addition, investigating the growth of micro colonies on agar gives the advantage of predicting the effects that can influence the colonial growth dynamics on actual food matrices.

CRediT authorship contribution statement

Lena Fritsch: Investigation, Formal analysis, Methodology, Visualization, Conceptualization, Writing - original draft. **Abirami Baleswaran:** Investigation, Formal analysis, Visualization. **Hélène Bergis:** Investigation, Resources, Writing - review & editing. **Adrienne Lintz:** Resources, Writing - review & editing. **Erwann Hamon:** Resources, Writing - review & editing. **Valérie Stahl:** Resources, Writing - review & editing, Supervision. **Jean-Christophe Augustin:** Writing - review & editing, Supervision. **Laurent Guillier:** Conceptualization, Writing - original draft, Methodology, Formal analysis, Supervision.

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